REVIEW

Human neutrophil elastase and lung surfactant in acute respiratory distress syndrome

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ABSTRACT

Human Neutrophil Elastase (HNE) is one of the main proteases secreted into the alveolar space by infiltrated neutrophils during several inflammatory lung diseases such as cystic fibrosis, acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). Consequently, a number of therapeutic approaches based on the specific inhibition of HNE are currently under investigation. The present work reviews the physiopathological role of HNE in ALI/ARDS and its relationship to the pulmonary surfactant system, as well as the clinical potential of protease inhibitors in this setting. In spite of the complex physiopathology of these diseases, the available evidence points to a direct link between HNE and ALI/ARDS, with increased local concentrations of this protease in animal models of ALI as well as in patients. Furthermore, the unbalanced ratio of protease/endogenous inhibitors characteristic of these disorders has led to the pharmacological and clinical evaluation of HNE inhibitors, examining their addition to currently available exogenous surfactant with promising results.

Keywords: human neutrophil elastase, acute lung injury, lung surfactant, inhibitors, ARDS

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RESUMEN

Elastasa de neutrófilos humana y surfactante pulmonar en el síndrome de distrés respiratorio agudo. En el desarrollo de algunas enfermedades inflamatorias pulmonares, tales como la fibrosis cística, el daño agudo del pulmón (ALI, del inglés Acute Lung Injury) y el síndrome de distrés respiratorio agudo (SDRA), se liberan numerosas proteasas a partir de neutrófilos infiltrados en el espacio alveolar; entre ellas se distingue la elastasa de neutrófilos humana (ENH). Con el objetivo de inhibir la actividad elastolítica, se han desarrollado estrategias terapéuticas. Este artículo revisa algunas de las funciones fisiopatológicas de la ENH en el ALI/SDRA, y su relación con el sistema surfactante pulmonar, así como el potencial terapéutico de los inhibidores de proteasas. Los resultados postulan que, aunque la fisiopatología de estas enfermedades es compleja, muchos ensayos demuestran una relación directa entre la ENH y el ALI/SDRA: se ha observado un incremento de la concentración de esta proteasa en modelos animales con ALI, así como en pacientes. Al mismo tiempo, debido al desequilibrio proteasa/inhibidores endógenos, se ha efectuado la evaluación farmacológica y se investiga la aplicación clínica de inhibidores de la ENH, así como su posible asociación con las preparaciones de surfactante pulmonar exógeno, y se tienen resultados prometedores.

Palabras clave: elastasa de neutrófilos humana, daño agudo del pulmón, surfactante pulmonar, inhibidores, SDRA

*I*ntroduction

Acute Lung Injury (ALI) and its more severe manifestation, Acute Respiratory distress syndrome (ARDS), are characterized by injuries to the lung parenchyma that compromise the respiratory function of the affected patients. Both are life-threatening diseases with a complex etiology involving inflammatory, infectious and oxidative processes, with no satisfactory therapy and a mortality rate of 40% even in highly developed countries [1].

One of the features of these syndromes is the development of biophysical and biochemical changes in the pulmonary surfactant that compromises the defensive mechanisms of the lung [2, 3]. These changes are caused by the release into the alveolar space of pro-inflammatory mediators, plasmatic proteins and proteases associated to the ongoing edema, as well as oxidative products that ultimately result into a severe inhibition of the surfactant system.

A major role in this process is played by neutrophils infiltrated into the alveolar space. These cells secrete a number of serine proteases such as cathepsin G, proteinase 3 and, specially, human neutrophil elastase (HNE), known for its highly destructive effects in the surrounding tissue.

This work reviews some of the physiopathological roles of HNE in the context of ARDS and its effect on the pulmonary surfactant system, as well as the therapeutic potential of protease inhibitors *per se* or in combination with preparations of exogenous pulmonary surfactant.

*P*ulmonary surfactant and acute respiratory distress syndrome

Lung surfactant is composed mainly of lipids (90%) and proteins (about 10%), although by mass only 6-8% of the latter are specifically associated to the

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surfactant. Approximately 80 to 85% of the lipid weight fraction is formed by phospholipids, which can be further decomposed into 75% phosphatidylcholine, 10% phosphatidylglycerol, 5% phosphatidylserine plus phosphatidyl inositol, and less than 5% sphingomyelin. Dipalmitoylphosphatidylcholine(DPPC) representing nearly half of the phosphatidyl choline content, thus constituting the main component of pulmonary surfactant. The major neutral lipid of this fluid is cholesterol, at proportions ranging from 5 to 10% of total lipids. In addition to these components, the surfactant has an array of minor components such as other phospholipids, glycerides, free fatty acids, lysophospholipids and glycolipids [4]. There are 4 major molecules in the protein fraction, denominated (in chronological order following their discovery dates) SP-A, SP-B, SP-C and SP-D [5]. SP-A and SP-D are water soluble, and the remaining two (SP-B and SP-C) are hydrophobic.

The main role of the pulmonary surfactant is to reduce work respiratory by decreasing surface tension at the air-liquid interface of the alveolus, in addition to other functions such as stabilizing the respiratory tract, enhancing mucociliary transport, preventing the appearance of edema, and contributing to the defense against pathogens [6, 7].

The hydrophilic proteins of the pulmonary surfactant (SP-A and SP-D) belong to the family of collectins, which includes molecules with a mannose-binding C type lectin domain associated to a collagen portion. The members of this family are major modulators of the innate immune system and, not surprisingly, SP-A and SP-D play a major role in pulmonary defense, functioning as opsonins by binding a number of microorganisms or pathogen-derived components and subsequently mediating microbial agglutination. The lung collectins, additionally, are inhibitory *per se* for bacterial growth [8].

ARDS was first described by Ashbaugh (1967) in twelve adult patients deceased from respiratory failure [9]. A large number of studies followed this initial report, detailing the epidemiology, physiopathology and therapy of this disorder. The European-American Consensus Conference on ARDS (1994), which updated and normalized a number of studies on this syndrome, defined an acute respiratory failure due to injuries to the pulmonary parenchyma as the manifestation of a number of disorders, such as acute respiratory failure (ARF), ALI and ARDS [10]. Although ARDS-related deaths have decreased, it still remains a significant health problem with a 40% mortality rate [1].

ALI/ARDS can be caused by different affections; the most common causes are infections due to a primary pulmonary injury, such as pneumonia, or through a systemic route, due to *e.g.* a sepsis. ALI-ARDS can also be caused by massive trauma, multiple blood transfusions and pancreatitis, in addition to direct lung injuries such as those resulting from gastric inhalation or from breathing toxic gases [8]. Several studies have shown an increase in pro- and anti-inflammatory mediators in the broncho-alveolar lavage of these patients. Although the release of these mediators may be part of a protective response during the early stages of the disorder, such a disequilibrium, if maintained in time, often leads to progressive lung dysfunction and eventually, to multiorgan failure and death [11]. Some of the changes resulting from this clinical inflammation are hypoxemia, infiltrates, and reductions in pulmonary flexibility.

ARDS is associated with biochemical and biophysical alterations of the pulmonary surfactant system [12], such as increased protein contents in broncho-alveolar lavage, altered phospholipid and fatty acids profiles [13, 14], lower SP-A and SP-B concentrations [15] and low levels of large aggregates, which constitute the biophysically active form of the surfactant. These changes lead to a marked reduction of the surface activity of the surfactant at the air-liquid interface of the alveolus, compounded by the degradation of essential components of the surfactant by inflammatory mediators (such as phospholipases and proteases, including HNE) and the inhibition of surfactant function by plasma proteins; these factors ultimately lead to the loss of alveolar stability and a concomitant, severe reduction in gas exchange. Consequently, the administration of surfactant preparations to ALI/ARDS patients has been proposed as a potential therapeutic alternative for this disorder.

The presence of pulmonary surfactant is absolutely essential for life, and its absence, deficiency or inactivation is associated with severe pulmonary disease as evidenced by disorders such as neonatal respiratory distress syndrome (NRDS), ALI and ARDS [2]. A major turning point in the therapy of this patient group was the development and application, by ABBOTT Laboratories (USA), of a natural bovine-derived pulmonary surfactant preparation trademarked under the name SURVANTA® at the beginning of the eighties [16]. This innovation was soon imitated by a number of pharmaceutical companies that developed and marketed their own exogenous natural surfactants, such as SURFACTEN® (Tokoyo Tanabe, Japan), ALVEOFACT® (Boehringer Ingelheim, Germany), CUROSURF® (Chiesi Pharmaceuticals, Italia), INFASURF® (Forest Laboratories, USA) and BLES® (BLES Biochem, Canada). "Besides these products, Cuba has developed a therapeutic preparation trade name, Surfacen®", is another recent example of such product [17]. In general, these natural surfactants have a biochemical composition characterized by a high phospholipid content (particularly phosphatidyl choline and its palmitic acid-saturated product, DPPC) and a relatively large proportion of anionic phospholipids (phosphatidyl glycerol and phosphatidyl inositol) in comparison to other phospholipid species, as well as by the presence of the hydrophobic proteins SP-B and SP-C [4] to the detriment of SP-A and SP-D, which are lost during the manufacturing process.

The use of exogenous pulmonary surfactant preparations has become established practice during the treatment of NRDS patients [7, 18]. In ARDS, however, the insufficiency in endogenous surfactant is not a primary deficiency due to an immature neonate lung, but a secondary pulmonary dysfunction arising from the inactivation of the surfactant by a clinical inflammatory process with a very complex etiology [19]. Therefore, the use of exogenous surfactant for the therapy of ARDS remains controversial, and still awaits the development of new clinical surfactants able to withstand the challenges posed by the damaged and

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inflamed lungs of adult patients. It should be noticed, however, that the current natural exogenous surfactant preparations leave ample room for ARDS-directed optimizations. One of these optimizations -the inclusion of additional active principles, such as HNE inhibitors- is discussed below.

Effect of the current exogenous pulmonary surfactant preparations on HNE activity

The effect of different preparations of exogenous pulmonary surfactant on the *in vitro* modulation of activated neutrophils has been previously studied with varying results. For example, EXOSURF® (a synthetic preparation composed of DPPC, cetyl alcohol and tyloxapol) induced elastase release from polymorphonuclear cells challenged with three elastase inductors. However, two other natural preparations (CUROSURF® and SURVANTA®) caused the opposite effect, *i.e.* a dose-dependant inhibition of HNE release, and the behavior of another clinical surfactant (Alveofact®) depended on both dose and the specific stimulus, increasing HNE release at low concentrations but inhibiting it at higher dosages (although this inhibitory effect was considerably weaker than that of CUROSURF® and SURVANTA®) [20]. The results imply that the specific biochemical composition of these preparations can have a large impact on their pharmacological effect [21].

Biological role of HNE and endogenous inhibitors

HNE is an important player in the innate immune response, where it hydrolyzes the peptidoglycan cell wall of Gram-negative bacteria and participates in the degradation of immune complexes phagocyted by polymorphonuclear leukocytes [22]. Additionally, this enzyme has probably a role in leukocyte migration from the circulatory system to the surrounding tissues [23].

The proteolytic activity of HNE is controlled, however, by multiple endogenous inhibitory proteins (Table 1). These inhibitors are found in the circulation or localized to specific cells or tissues at high concentrations, and fulfill an important function by protecting them against uncontrolled degradation by highly active proteases. In order of importance following their abundance and specific characteristics, some of these inhibitors are: α_1-antitrypsin (α_1-AT) or α_1 protease inhibitor; α_2-macroglobulin; secretory leukocyte peptidase inhibitor (SLPI), pre-elafin and elafin; monocyte/neutrophil elastase inhibitor (M/NEI) and PI9 (Protease inhibitor 9) [24, 25].

The α1-AT molecule has a molecular weight of 55 kDa, and constitutes the most important irreversible HNE inhibitor due to both its concentration (54 µmol/L in plasma) and fast kinetics of action (k_{on} 6 x10^7 $mol^{-1}Ls^{-1}$). It is an inhibitor from the serpin family, along with M/NEI and PI9 [25-27]. α1-AT is responsible for 92% of the HNE inhibitory activity in plasma, with the remaining portion being attributed to α2-macroglobulin. These two major inhibitors have, however, very different mechanisms of action: α1-AT inhibits HNE irreversibly by forming a stable acyl-enzyme complex at the catalytic site, whereas α2-macroglobulin forms a molecular cage that isolates the protease, excluding almost all substrates except some native proteins and substrates small enough to reach through the active site [28]. The physiological relevance of the remaining proteolytic activity of HNE entrapped into α2-macroglobulin complexes, if any, has not been elucidated; however, some evidences suggest that it is partially responsible for the tissue degradation observed in pulmonary emphysema [27].

SLPI is a much smaller (11.7 kDa) protein that constitutes the most important HNE inhibitor of the upper respiratory tract; it is synthesized and secreted by a wide array of cell types such as epithelial and bronchial gland cells [33, 34]. HNE is the target protease of SLPI, which inhibits the former reversibly with a Ki of 4 x 10^{-11} mol/L [35]. SLPI and elafin belong to the chelonianin family, containing canonical serine protease inhibitors [36]. There are two domains in SLPI; the inhibitory activity is localized in the carboxyl domain, whereas the amino-terminal domain has antibacterial activity against both Gram-negative and positive microorganism [34]. Compared to α1-AT and α2-macroglobulin, SLPI has easier access to sterically restricted locations, conferring this molecule an important role in the protection against tissue damage mediated by HNE [37].

Elafin, a 6.0 kDa protein isolated from lung secretions [20, 38] or human epithelium [39], is proteolytically released from a larger trappin-2 or pre-elafin precursor. Its amino-terminal region is characterized by the presence of a repetitive aminoacid motif, which apparently mediates binding of elafin to proteinaceous components of the lung in order to restrict its diffusion to the target site. Elafin is a reversible inhibitor for HNE (Ki 2 x 10^{-10} mol/L) [40] as well as for proteinase 3 (another serine protease secreted by neutrophils) [40]. In a hamster model of ALI, the intratracheal administration of recombinant human

Tabla 1. Inhibidores endógenos de la ENH

Name	Localization	Inhibitor family	Physiological role and therapeutic importance	Reference
α_1 -AT (α-1-antitripsin)	Plasma and large number of tissues	Serpins	Main physiological inhibitor of HNE A hereditary deficiency in this inhibitor results in disorders such as panacinar emphysema and cystic fibrosis	28
SLPI (secretory leukocyte protease inhibitor)	Epithelial cells, macrophages and neutrophils	Chelonianins	Inhibits HNE and cathepsin G Proven antiviral, bactericidal and antifungal activity Modulates a number of anti-inflammatory activities	29
Pre-elafin and elafin (skin-derived antileuko-protease inhibitor)	Bronchial secretions and skin	Chelonianins	Inhibits HNE and protease-3 Reduces the inflammatory response mediated by leukocytes and cytokines	30, 31
M/NEI (Monocite/ neutrophil elastase inhibitor)	Macrophages and neutrophils	Serpins	Inhibits HNE, pancreatic elastase and proteasa-3 In recombinant form is a potential treatment for cystic fibrosis	26
PI9 (human proteinase inhibitor 9)	Placenta, lung and cytotoxic lymphocytes	Serpins	Inhibits HNE and cathepsin G	32

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pre-elafin inhibits lung hemorrhages; synthetic elafin, however, was not effective in this model [41].

M/NEI, also known as SERPINB1, is a member of the serpin family and one of the most efficient inhibitors of HNE, cathepsin G and protease-3. SERPINB1 is extensively expressed at high concentration in the cytoplasm of neutrophils. A role in the preservation of the cellular and molecular components responsible for the defense against *Pseudomonas aeruginosa* was recently shown for SERPINB1, evidenced by the inability of mice devoid of this inhibitor to clear this bacterium from the lung and avoid the subsequent systemic infection. The immune system defect in *serpinb1*-negative mice leads to an increased necrosis rate in neutrophils, a reduction in the number of phagocytes, and an increased activity of serine proteases from neutrophils in the lung, resulting in the proteolysis of SP-D [42]. The most recent studies suggest that M/NEI can protect the airways by regulating the excess proteolytic activity associated with pulmonary inflammatory disorders, and in general the serpin family inhibitors have potentially promising therapeutic applications [43].

The physiological role of endogenous HNE inhibitors is to provide an anti-elastase activity that counteracts the potentially damaging effect of this enzyme in the surrounding tissues. However, in spite of the presence of large amounts of α1-AT, HNE in purulent fluids is known to degrade a large number of native proteins [27]. HNE escapes regulation by endogenous inhibitors due to a number of causes, including the large sizes of α1-AT and α2-macroglobulin, which hinder their full access to some sites of the neutrophil-tissue microenvironment as a result of stereochemical considerations. In such sites, the burden of proteolytic inhibition is shifted towards SLPI and elafin instead [27]. A second mechanism is the inactivation of α1-AT through myeloperoxidation, a process that also inactivates α2-macroglobulin and SLPI [27] where Met358 (at the reactive site of α1-AT) oxidizes to sulphoxide due to reactive oxygen species produced by activated neutrophils; and yet a third cause is the poor activity of these inhibitors towards surface-bound HNE on neutrophils and lung tissues, contrasting with their inhibitory activity on the free protease [37, 44]. Additionally, it is argued that the large accumulation of granules from human neutrophils provides a large amount of HNE, breaks the homeostatic elastase-inhibitor equilibrium and leads to acute tissue damage, as shown in patients suffering from α1-AT deficiency [46].

Effect of HNE on ALI/ARDS: therapeutic potential of HNE inhibitors

In the context of a number of respiratory diseases, HNE escapes its endogenous regulation mechanisms, altering pulmonary permeability and inducing the release of pro-inflammatory cytokines. An increase in HNE levels in both animal models of ALI and in clinical settings produces typical ALI symptoms, and the topical or systemic administration of exogenous HNE reproduces these symptoms *in vivo* and for *in vitro* markers. Additionally, inhibiting the increased HNE activity reduces ALI symptoms in animal models [27, 46].

The HNE-mediated damage occurs through the action of the enzyme on several potential targets at the lung. For instance, it has been shown that HNE inactivates or degrades antimicrobial factors of the liquid surface of the airways. Another target is the constituting proteins of the pulmonary surfactant: the adsorption of the surfactant to the air-liquid interface decreases in the presence of HNE, altering the surfactant role of this fluid *in vivo*. This effect has been proven to depend on HNE-mediated proteolytic degradation of SP-A, SP-B and SP-C [47], and was recently confirmed on cystic fibrosis patients where HNE and cathepsin G were detected in broncho-alveolar lavage. The incubation of purified SP-A with these lavage caused its degradation, and the addition of exogenous cathepsin G or HNE to broncho-alveolar lavage from normal persons resulted in the dose-dependent degradation of endogenous SP-A. This degradation was abolished by the addition of two inhibitors: M/NEI and diisopropyl fluorophosphate [48].

In vitro assays have also shown that SP-D degrades upon incubation with the neutrophil proteases NHE, protease 3 and cathepsin G through the specific hydrolysis of a conserved region in the carbohydrate-binding domain of this protein. These results were confirmed in an experimental mouse model of bacterial pneumonia [49]. Based on these evidences, it is safe to conclude that the serine proteases of neutrophils, especially NHE and cathepsin G, play a significant role in the proteolytic degradation of the pulmonary surfactant, and therefore are detrimental for the innate antimicrobial defense of the lungs.

Part of the complex lifecycle of the pulmonary surfactant is its conversion from large aggregates of active surface (responsible for the excellent biophysical properties of this fluid) into smaller inactive aggregates [50]. This process, which is part of the normal extracellular metabolism of the surfactant, is probably regulated by a serine protease known as the surfactant convertase which is produced by alveolar macrophages and type II lung cells [51], and depends on the expansion-compression process at the air-liquid interface [52]. Although the surfactant convertase is not the only enzyme involved in this process, the available evidence suggests that its activity is highly specific for the conversion of surfactant types. The aminoacid sequence of the convertase has been determined, and shows that it is a serine carboxylesterase [53]. Additionally, the surfactant convertase is sensitive to α1-AT-mediated inhibition, according to the data from *in vitro* and *in vivo* studies.

The effect of SURVANTA® (a commercial preparation of exogenous surfactant) as well as phospholipid and/or synthetic protein mixtures representing the main components of the pulmonary surfactant together with α1-AT have been evaluated in a surfactant-deficient rat model. The results showed a significantly improved rate of oxygenation, associated with increased numbers of large surfactant aggregates possibly caused by an inhibition of surfactant convertase [54]; suggesting a positive role for α1-AT in the prevention of surfactant degradation in the lung. Other inhibitors have also been shown to modulate the process of surfactant aggregate conversion [55], and given the potential pharmacological activity of HNE inhibitors

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as additives for clinical surfactant preparations, such a course of action should be considered for clinical evaluation during the therapy of ALI/ARDS.

The importance of inflammatory process in the etiology of lung diseases and the widespread involvement of HNE in their genesis has steered the development of candidate drugs for these disorders towards the search for specific HNE inhibitors [56]. Table 2 illustrates the main synthetic and recombinant HNE inhibitors developed to date which have reached some state of clinical evaluation. One of the most successful cases is represented by Sivelestat, a synthetic, highly specific HNE inhibitor. The intravenous infusion of this inhibitor in a hamster model of severe ALI induced by instillation of hydrochloric acid and by *Streptococcus pneumoniae* results in a significant improvement of injury markers in broncho-alveolar lavage and in pO_2, associated with a successful inhibition of HNE activity and a decrease in mortality [57, 58]. This same group used a model of phorbol myristate acetate-induced acute lung injury in conscious rabbits to show that the application of Sivelestat inhibited HNE activity by 60 to 90% while at the same time attenuating hemorrhage and decreasing protein contents at the lung [59]. Sivelestat has also been shown to effectively decrease the values of inflammation parameters, pulmonary edema and acute damage in multiple animal models [60].

Clinical trials have demonstrated that the therapeutic use of Sivelestat results in a significant improvement of oxygenation, reducing the time during which patients remain connected to a ventilator in intensive care units. Sivelestat therapy, however, did not provide a significant decrease in mortality for patients with respiratory distress syndrome associated to the systemic inflammatory response syndrome [61, 62]. The small molecular weight of Sivelestat when compared to the endogenous protease inhibitors mean that its delivery to the inflammation site is easier and more effective, relieving the clinical symptoms of ALI [57, 63].

Still, a research is needed to solve the enigma in pulmonary surfactant and in the optimization of synthetic or recombinant HNE inhibitors for their use in the complex scenario of respiratory diseases. The combination of both preparations may represent an interesting alternative with a large therapeutic potential in the treatment of ALI and ARDS.

Table 2. Synthetic and recombinant inhibitors of HNE. Updated clinical development status for respiratory diseases

Name	Indication	Clinical development status	Manufacturer
Sivelestat, Elaspol, ONO 5046 o LY544349	Treatment of acute lung injury associated to systemic inflammatory response syndrome	Marketed in Japan (2002) and South Korea (2006)	Ono Pharmaceutical Co., Ltd. and Dong-A Pharmaceutical Co.
Midesteine	Chronic obstructive pulmonary disease (COPD)	Awaiting regulatory approval(Italia)	Medea Research
AE-3763	Chronic obstructive pulmonary disease (COPD)	Pre-clinical	Dainippon
R-448	Chronic obstructive pulmonary disease (COPD)	Phase I	Roche
Elafin Proteo	Pulmonary arterial hypertension and chronic thromboembolic pulmonary hypertension	Phase II	Proteo Inc. and Proteo Biotech AG
ADC 7878	Cystic fibrosis	Pre-clinical	Argenta Discovery Ltd. (Private)
AZD9668	Chronic obstructive pulmonary disease (COPD)	Phase II	AstraZeneca Plc. (AZN)
AGTC-0106	Emphysema (α1-AT deficiency)	Phase I	Applied Genetic Technologies Corporation (AGTC)
Respriva	Emphysema (α1-AT deficiency)	Phase II	Arriva Pharmaceuticals Inc.(Private)

Conclusion

Proteases are obvious targets for inhibitor-based therapies. They are involved in the physiopathology of infectious inflammatory diseases such as ALI and ARDS, although a large number of questions regarding their specific role remain unanswered. The main obstacles of the clinical application of inhibitors in this setting are the difficulties inherent to the obtention of drugs that eliminate or neutralize the pathogenic effects of HNE without interfering with its normal physiological role or eliciting unwanted side effects. The number of studies examining the combination of exogenous pulmonary surfactant preparations with protease inhibitors is still small, and therefore this alternative remains a potentially rewarding research avenue for the development of therapies against these life-threatening lung disorders.

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